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13. Abstract ***(Maximum 200 words)*** *(abstract should contain no proprietary or conficential information)*

One of the earliest common changes in the development of breast cancer occurs when some breast epithelial cells begin to grow and proliferate independently of estradiol (E2). We are conducting studies on model breast epithelial cell lines: MCF7 cells, whose proliferation is dependent on estradiol; and LCC1 cells, a cell line derived from MCF7 with an acquired E2 independence for growth. We continue to apply proteomics techniques (two dimensional electrophoresis, image analysis and protein identification by mass spectrometry) to characterize broadly the patterns of protein expression in these two cell lines and their regulation by estradiol. We have identified several dozens of proteins in MCF7 cells which are up- or down-regulated in association with E2-controlled proliferation. Many of these are seen constitutively altered in the LCC1 cells grown in the absence of E2. While there is little or no effect of E2 on the proliferation of LCC1 cells, we find many proteins whose levels are altered by the addition of E2. Our results are consistent with the hypothesis that E2-dependent MCF7 cells undergo apoptosis upon removal of E2 while the LCC1 cells have lost the ability to induce apoptosis upon removal of E2 and thereby display E2-independent growth.

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INTRODUCTION

One of the earliest common changes in the development of breast cancer occurs when some breast epithelial cells begin to grow and proliferate independently of estradiol (E2). We are conducting studies on model breast epithelial cell lines: MCF7 cells, whose proliferation is dependent on estradiol; and LCC1 cells, a cell line derived from MCF7 with an acquired E2 independence for growth. We continue to apply proteomics techniques (two dimensional electrophoresis, image analysis and protein identification by mass spectrometry) to characterize broadly the patterns of protein expression in these two cell lines and their regulation by estradiol. We have identified several dozens of proteins in MCF7 cells which are up- or down-regulated in association with E2-controlled proliferation. Many of these are seen constitutively altered in the LCC1 cells grown in the absence of E2. While there is little or no effect of E2 on the proliferation of LCC1 cells, we find many proteins whose levels are altered by the addition of E2. Our results are consistent with the hypothesis that E2-dependent MCF7 cells undergo apoptosis upon removal of E2 while the LCC1 cells have lost the ability to induce apoptosis upon removal of E2 and thereby display E2-independent growth.

BODY

Representative silver-stained 2D gels of total proteins isolated from MCF7 cells minus 17-β-estradiol (E2) covering three pH ranges are shown in Figure 1. Overlapping pH ranges are used to increase the number of considered protein features for a given complex sample. These images represent three of the over 400 such gels that have been run during the first two years of this work. We have had excellent reproducibility in the gel images obtained for total cell proteins isolated from the various cell conditions tested: MCF7 cells plus or minus E2 and LCC1 cells plus or minus E2. Numerous attempts at subcellular fractionation and membrane isolation, while largely successful as judged by microscopy and 2D-gel based proteome analysis, were not sufficiently reproducible to allow precise analysis of differential protein expression.

Most of the initial time in this study was spent replicating Dr. Clarke's conditions as closely as possible by harvesting the cells 24 hours after addition of E2 to the medium. The effect of E2 on the proteomes of the MCF7 cells was virtually non-existent. That is to say the proteins detected in 2D gels from estrogen-stimulated cells were virtually indistinguishable from those of unstimulated cells. More consistent and numerous changes were observed when comparing MCF7 with LCC1 cells. As mentioned in the previous report (May 2001) we find a pronounced 1 day lag between the time of E2 addition and detection of increased growth of MCF7 cells. All studies in the present report were after re-stimulation of the cells with E2 for 48 hours whereby we observe many more significant and reproducible proteome changes (summarized below).

We have switched from analyzing our gels using the Phoretix 2D gel analysis software (nonlinear Dynamics) to using the more advanced Progenesis gel analysis software from the same vendor to take advantage of the enhanced spot detection, image warping, and database features of this new software. We have completed the analysis of the pH 5-8 gels and are presently analyzing the ph 3-6, 4-7, and 7-10 gel images. Spot detection is illustrated in Figure 2 and image warping and spot matching is illustrated in Figure 3. We use averaged gels constructed from multiple individual gels for a given condition. For a protein feature to be considered it must appear and be matched in at least 4 out of 6 individual gels. For pH 5-8 gels,

total number of spots satisfying this criterion for each condition are listed in Table 1. Table 2 summarizes the total protein changes observed upon addition of E2 to MCF7 and LCC1 cells and the effect of acquired E2 independent growth. Table 3 shows that there are very few E2-induced changes held in common between MCF7 and LCC1 cells whereas there are many changes held in common between the effect of E2 on MCF7 cells and the effect of acquired E2 independent growth (Table 4). The 73 proteins which increase (Tables 2 and 4) as a result of acquired E2 independent growth of LCC1 cells are potential markers for early detection of breast cancer. We are presently engaged in the process of evaluating each of these proteins on an individual basis.

We have identified most of the hundreds of protein alluded to in tables 2 through 4 by a combination of MALDI TOF MS peptide mass mapping and LC ESI MSMS analysis. The most consistent pattern emerging is a strong correlation with cell proliferation of proteins associated with polynucleotide synthesis and processing, protein synthesis, and nuclear transport. This is not surprising. There are also several changes observed in post-translationally modified protein species of nuclear lamins, several cytokeratins, and some chaperonins. One of the more interesting findings is that when E2 is removed from MCF7 cells and the cells cease to proliferate or even decrease in number, we find specific truncated forms of cytokeratins 18 and 19 appear (for example see Figures 4, 5 and 6). The full length cytokeratins 18 and 19 (not labeled) are found in the center left region of the gel (Figure 4) among the dark cluster of abundant poorly resolved proteins. Detailed sequence analysis suggests that all of these specific truncated forms are consistent with the action of pro-apoptotic caspases.

We are examining further the idea that acquired estradiol independent growth in LCC1 cells results from a loss of activation of apoptosis upon removal of estradiol. The results of cell shakeoff and reattachment assays we have done are consistent with induction of apoptosis by removal of E2 from MCF7 but not LCC1 cells. We are presently doing more accurate flow cytometry experiments to determine the proportions of cells in G2/M phase, G1/G0, and cells with sub-G0 DNA content (indicative of apoptotic cells) for each of our four conditions. We are also asking if apoptosis is constiuatively suppressed in LCC1 cells by looking at the phosphorylation status of Akt1. Many cell survival signals work by suppressing apoptosis through this pathway.

KEY RESEARCH ACCOMPLISHMENTS

Task 1. Set up 2D-electrophoresis system to analyze and compare the proteomes of MCF7 and MCF7/LCC1 breast cells and prepare initial 2D-gels for mass spectrometry protein identification. *(month 1-3)*

Completed in Year 1

Task 2. Implement software and techniques for producing a master gel pattern whereby changes in protein expression patterns among breast cell lines can be recognized with computer assistance. *(months 1-8)*

Completed in Year 1 and improved in year 2 (see body of report)

Task 3. Use mass spectrometry to sequence and identify remaining members of the set of abundant proteins that reflect the MCF7 response to estrogen and the MCF7/LCC1 acquisition of estrogen independence. *(months 2-4)*

Completed in year 2.

Task 4. Further elucidate the differences between MCF7 and MCF7/LCC1 cells and the response of these cells to estrogen to find those proteins that have eluded detection by virtue of their lower abundance *(months 6-18)* and/or previously unexplored isoelectric point range. *(months 9-21)*

Begun in year 1, extensive data collected in year 2, analysis will be completed in year 3.

Task 5. We will perform subcellular fractionation to characterize the proteomes of nuclear, soluble, and especially the membrane fractions. *(months 12-24)*

Begun in year 1, completed in year 2. This approach was judged too irreproducible for meaningful comparison of different cells grown under various conditions. The data are useful, however, for indicating which subcellular compartment a particular protein feature is associated with.

Task 6. Rapidly evolving improvements in mass spectrometry technology and database searching software will be implemented to improve sensitivity and to better sequence and identify newly selected proteins of lower abundance. *(months 1-24)*

We have continued to improve our general laboratory material handling practices allowing greater throughput and sensitivity in our mass spec analyses of protein features identified in gels. We have implemented a much more powerful image analysis package (Progenesis, Non-linear dynamics) than we were previously using. A new research grade MALDI TOF mass spectrometer (Reflex IV, Bruker Daltonics) was installed in year 2 allowing much more sensitive and accurate peptide mass mapping for protein identification. We have acquired and installed MASCOT (Matrix Science) for doing automated peptide mass map sequence database searching on a local server. This has allowed higher throughput than using web-based search engines. MASCOT also provides a complement to our SEQUEST database searches of LC-MSMS data.

REPORTABLE OUTCOMES

Not applicable

CONCLUSIONS

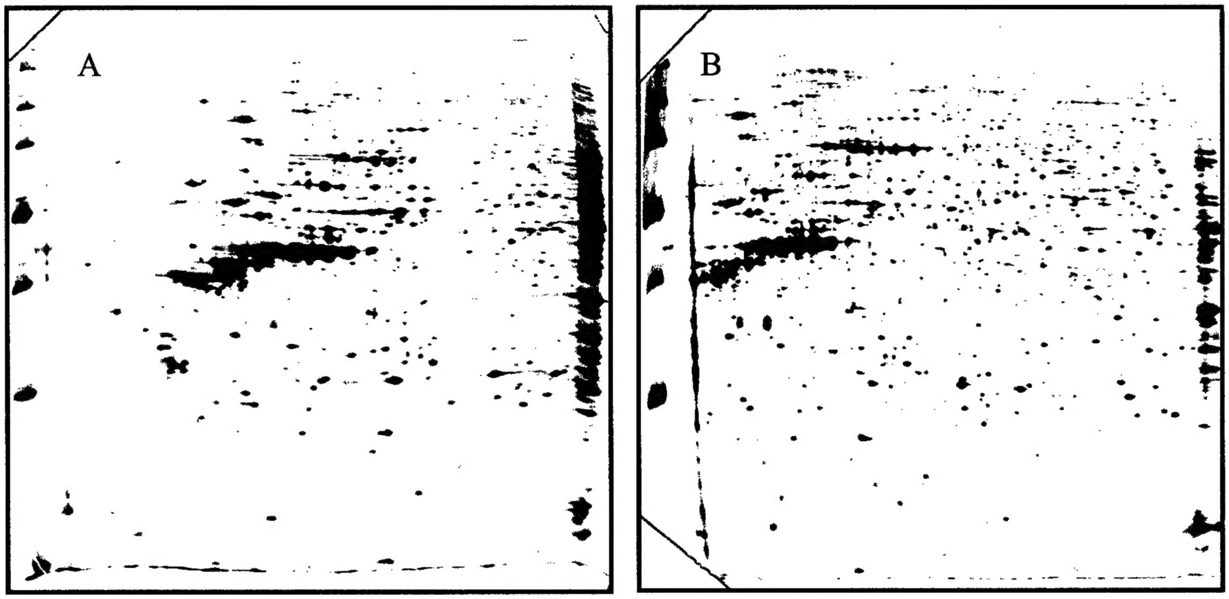
We have confirmed many of the expected E2-induced protein changes in MCF7 and LCC1 cells and have extended these observations to many newly identified protein markers associated with acquired estradiol independent growth. Estradiol appears to suppress apoptosis in dependent

MCF7 cells whereas apoptosis appears to be constituitively suppressed in the estradiol-independent LCC1 cells.

REFERENCES

Skaar TC. Prasad SC. Sharareh S. Lippman ME. Brunner N. Clarke R. Two-dimensional gel electrophoresis analyses identify nucleophosmin as an estrogen regulated protein associated with acquired estrogen-independence in human breast cancer cells. Journal of Steroid Biochemistry & Molecular Biology. 67(5-6):391-402, 1998

Figures and Tables.



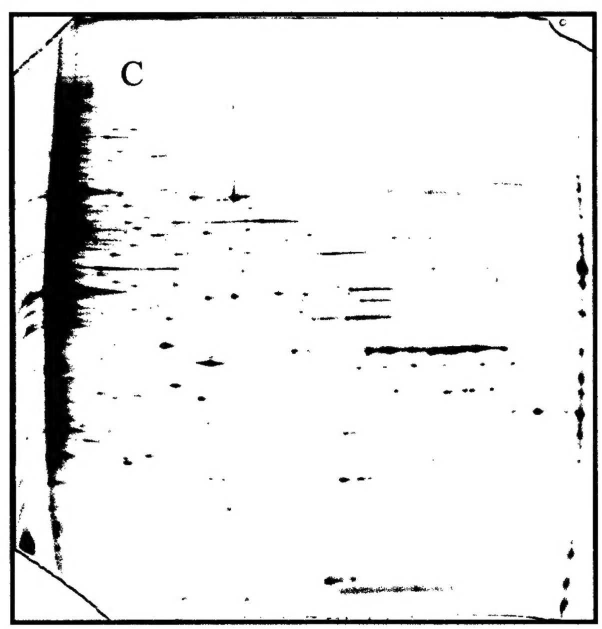


Figure 1. Representative silver stained gels of total cell proteins from MCF7 cells without estradiol. A. pH 4-7; B. pH 5-8; and C. pH 7-10. Gels from MCF7 cells in the presence of estradiol and LCC1 cells with or without estradiol are qualitatively similar.

Figure 2. Illustration of spot detection by Progenesis software. Extensive editing of spot detection was found to be necessary in order to rejoin inappropriately split spots and split inappropriately joined spots.

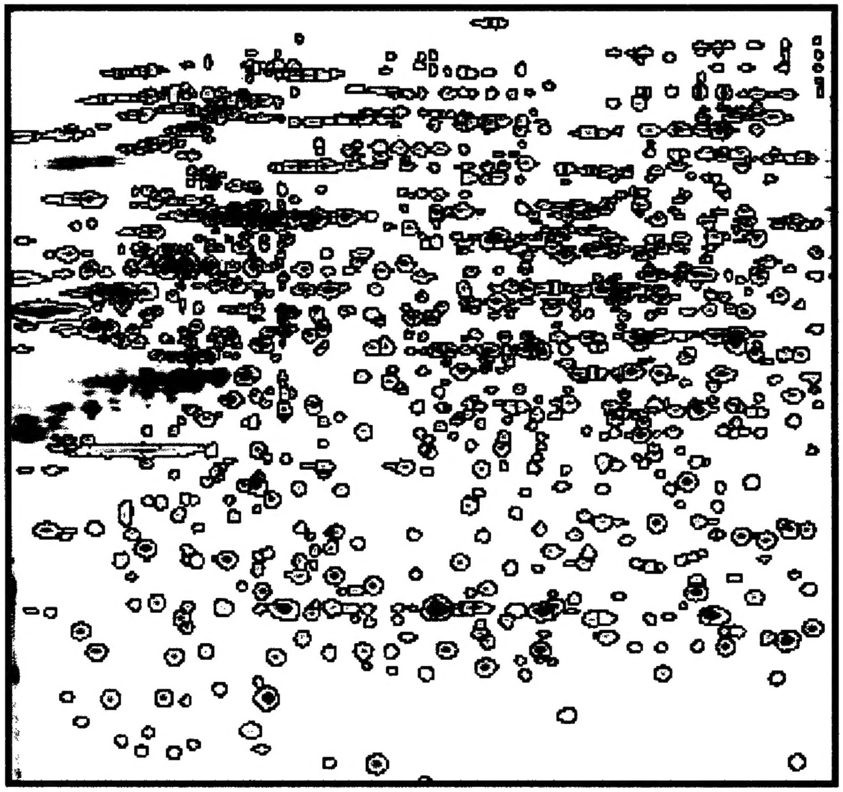
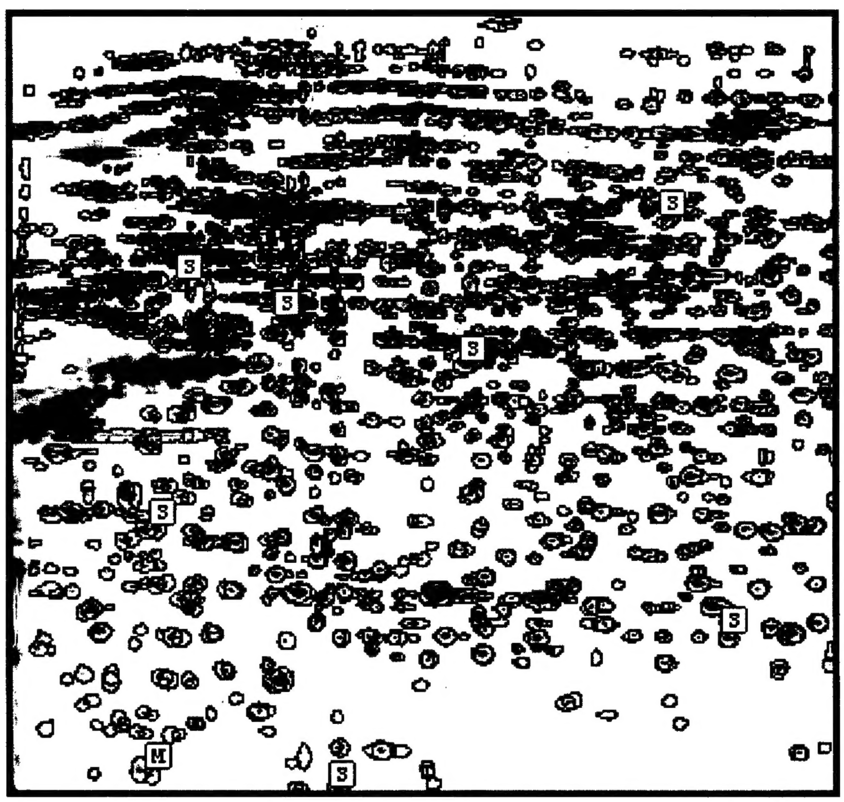


Figure 3. Illustration of gel image warping and spot matching procedure. User selected seeds for matching were chosen from highly reproducible local constellations of protein features. User seeds were invariably shown by mass spectrometry to be the same protein.



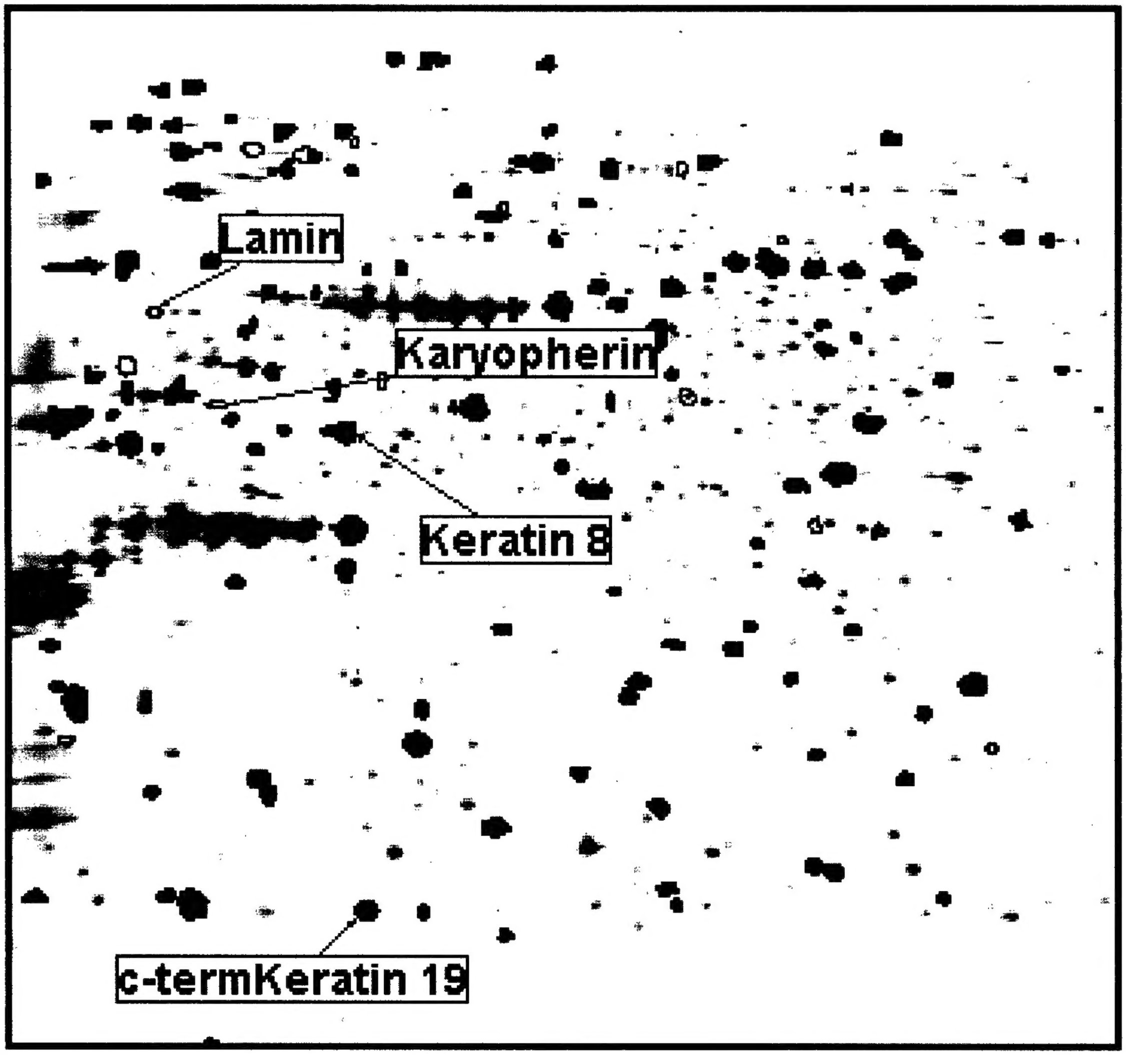


Figure 4. Gel image of MCF7 –E2 with E2-induced changes in MCF7 cells superimposed in color. Blue spots represent protein features not found in MCF7 +E2 but found in MCF7 –E2 (eg. C-term fragment of keratin 19). Green spots show a 2 fold increase upon removal of estradiol (eg Keratin 8) while yellow colored spots represent proteins decreased by the removal of estradiol (eg. Karyopherin, lamin).

Condition	Number of Unique Protein Features in Averaged Gels
MCF7 + E2	930
MCF7 - E2	814
LCC1 +E2	899
LCC1 –E2	891

Table 1. Summary of protein features comprising averaged gels for comparison across conditions. While 1200 to 1600 protein features were detected in each individual gel for each condition, these numbers represent protein features detected and matched in at least 4 out of every 6 gels for each condition.

Comparison	Increased	Decreased	Total change
MCF7 +E2 vs MCF7 – E2	45	29	74
LCC1 +E2 vs LCC1 – E2	13	47	60
LCC1 -E2 vs MCF7 – E2	73	51	124

Table 2. Effect of estradiol on MCF7 cells and on LCC1 cells. Shown are number of proteins showing greater than 4-fold changes in pH 5-8 2D gels.

Comparison	Increased	Decreased	Total change
MCF7 +E2 vs MCF7 – E2	44	26	70
Changes in common	1	3	4
LCC1 +E2 vs LCC1 – E2	12	44	56

Table 3. Effect of estradiol on MCF7 cells and on LCC1 cells. Shown are number of proteins with greater than 4-fold changes in pH 5-8 2D gels. The middle row shows those changes held in common between the two comparisons.

Comparison	Increased	Decreased	Total change
MCF7 +E2 vs MCF7 – E2	23	15	38
Changes in common	**22**	14	36
LCC1 –E2 vs MCF7 –E2	**51**	37	88

Table 4. Effect of estradiol on MCF7 cells and effect of acquired estradiol independence of LCC1 cells. Shown are number of proteins with greater than 4-fold changes in pH 5-8 2D gels.The middle row shows those changes held in common between the two comparisons.

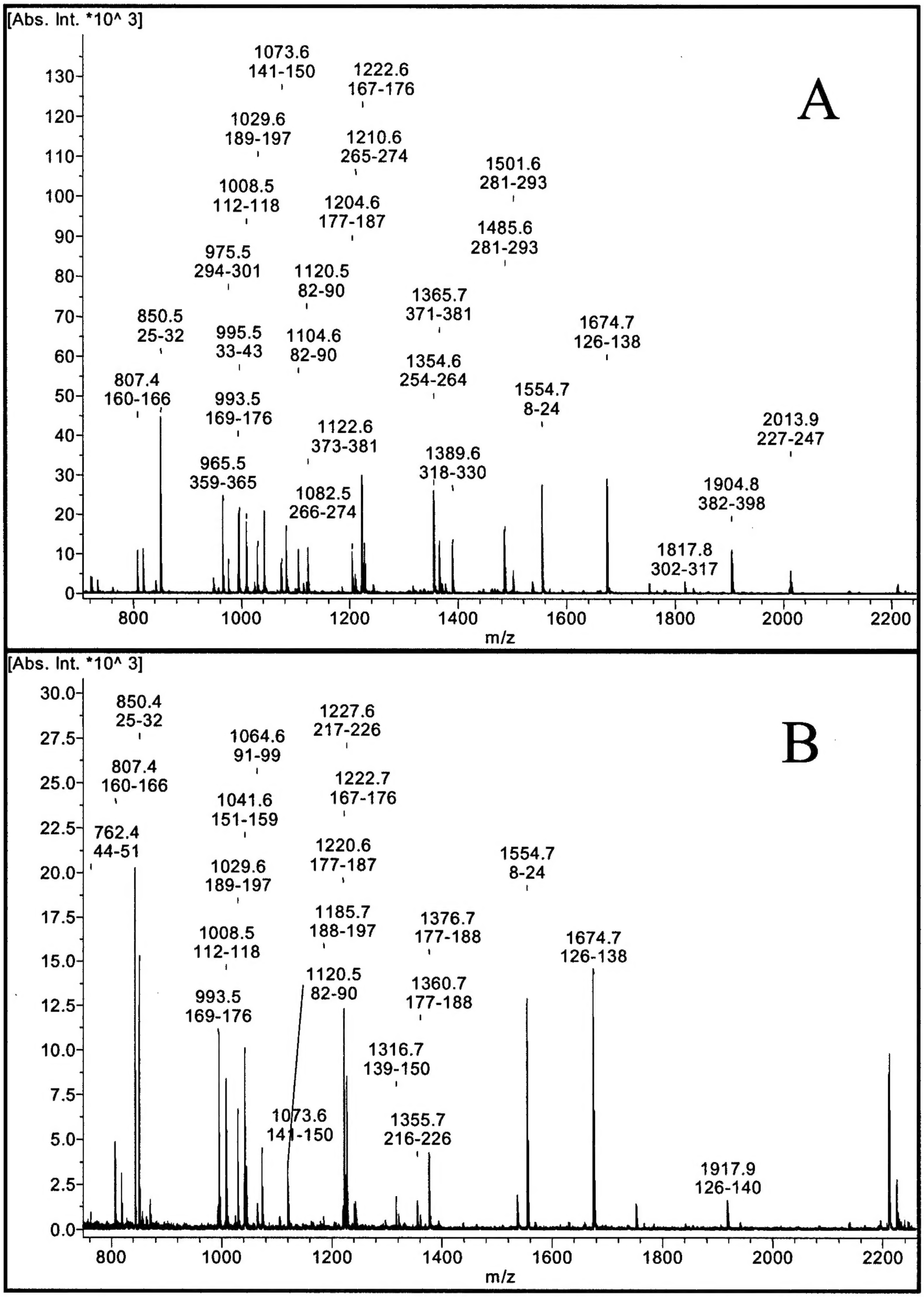


Figure 5. Tryptic peptide mass maps of full length (A) and proteolytically processed (B) keratin 19. The 2D gel spot giving rise to the processed form is only seen in MCF7 cells in the absence of estradiol (Figure 4).

A

Match to: **KRHU9**; Score: **338**

keratin 19, type I, cytoskeletal - human

Nominal mass (M_r): **44065**; Calculated pI value: **5.04**

Fixed modifications: Carbamidomethyl (C)

Variable modifications: Oxidation (M)

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P

Number of mass values searched: **35**

Number of mass values matched: **26**

Sequence Coverage: **59%**

Matched peptides shown in **Bold Red**

1 MTSYSYR**QSS ATSSFGGLGG GSVRFGPGVA FRAPSIHGGS GGR**GVSVSSA

51 RFVSSSSSGG YGGGYGGVLT ASDGLLAGNE K**LTMQNLNDR** LASYLDKVRA

101 LEAANGELEV K**IRDWYQK**QG PGPSR**DYSHY YTTIQDLR**DK **ILGATIENSR**

151 IVLQIDNAR**L AADDFRTKFE TEQALRMSVE ADINGLRRVL DELTLAR**TDL

201 EMQIEGLKEE LAYLKKNHEE EISTLR**GQVG GQVSVEVDSA PGTDLAK**ILS

251 DMR**SQYEVMA EQNRKDAEAW FTSR**TEELNR **EVAGHTEQLQ MSRSEVTDLR**

301 **RTLQGLEIEL QSQLSMKAAL EDTLAETEAR** FGAQLAHIQA LISGIEAQLG

351 DVRADSER**QN QEYQR**LMDIK **SRLEQEIATY RSLLEGQEDH YNNLSASK**VL

401

B

Match to: **KRHU9**; Score: **251**

keratin 19, type I, cytoskeletal - human

Nominal mass (M_r): **44065**; Calculated pI value: **5.04**

Fixed modifications: Carbamidomethyl (C)

Variable modifications: Oxidation (M)

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P

Number of mass values searched: **46**

Number of mass values matched: **23**

Sequence Coverage: **44%**

Matched peptides shown in **Bold Red**

1 MTSYSYR**QSS ATSSFGGLGG GSVRFGPGVA FR**APSIHGGS GGR**GVSVSSA**

51 **RFVSSSSSGG YGGGYGGVLT ASDGLLAGNE KLTMQNLNDR LASYLDKVR**A

101 LEAANGELEV K**IRDWYQKQG PGPSRDYSHY YTTIQDLRDK ILGATIENSR**

151 **IVLQIDNARL AADDFRTKFE TEQALRMSVE ADINGLRRVL DELTLAR**TDL

201 EMQIEGLKEE LAYLK**KNHEE EISTLR**GQVG GQVS**VEVD**SA PGTDLAKILS

251 DMRSQYEVMA EQNRKDAEAW FTSRTEELNR EVAGHTEQLQ MSRSEVTDLR

301 RTLQGLEIEL QSQLSMKAAL EDTLAETEAR FGAQLAHIQA LISGIEAQLG

351 DVRADSERQN QEYQRLMDIK SRLEQEIATY RSLLEGQEDH YNNLSASKVL

401

Caspase consensus cleavage site: [ILV]ExD, shown in **Bold Blue**

Figure 6. Sequence coverage of full length (A) and proteolytically processed (B) keratin 19 corresponding to the peptide mass maps shown in Figure 5. The experimental pI and mass values match the calculated values in A but not in B.